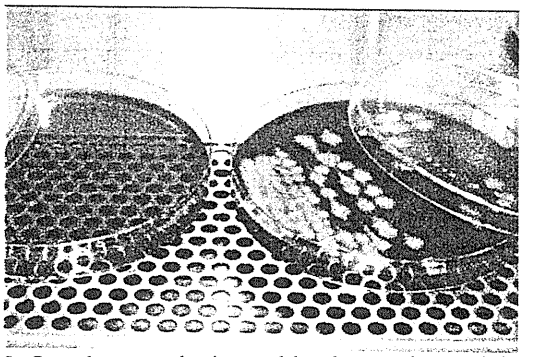

Fig. 2. *B.anthracis* colonies on blood agar plates (right site)

However, all the soil samples were negative. PCRs were analyzed with extracted DNA from *B.anthracis* strains, clinical and soil samples. Seven clinical samples were positive with PCRs by using both WHO and NIID protocols (one of the results is showed in Fig.3).

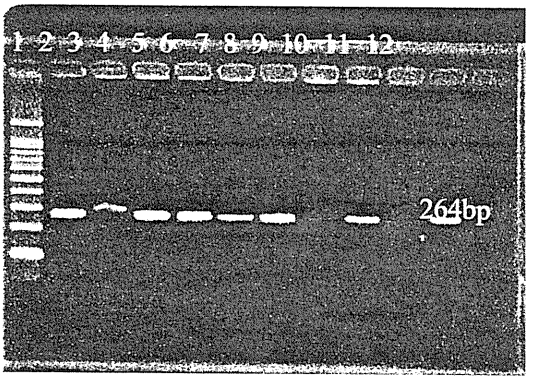

Fig.3. Identification of *B.antracis* by PCR with MO11/MO12 primers

Lane 1: molecular size marker (invitrogen)
Lane 2 – 10: clinical samples
*Lane 4& 5: same sample
Lane 11: positive control
Lane 12: negative control

The artificial infected soil sample was also positive by conventional PCR using Ba813 R1/R2 primers (showed in fig.4)

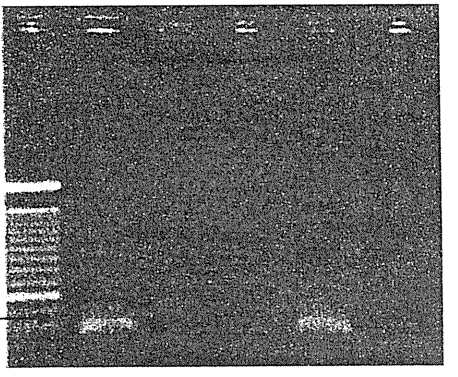

Fig.4. PCR result for detection of *B.anthracis* from artificial infected soil sample by using *Ba813* R1/R2 primers

Lane 1: molecular size marker (invitrogen)
Lane 2: 17JB
Lane 3-4: Soil samples
Lane 5: Soil + spores
Lane 6: Negative

6.10 Discussions

With long history, anthrax is considered one of the most dangerous zoonotic infectious diseases and the agent has been used for biological weapon. Therefore, continuous surveillance for anthrax is essential to prevent the outbreak disease and minimize its threat. To achieve that goal a rapid and accurate detection methods for *B.anthracis* from a variety of different products is very important.

In the published studies, PCRs with the specific nucleic acid probe for *B.athracis* (no cross-reactivity with other bacterial specific) have been reported. The primers often use to amplify the target genes such as *pag, cag, lef*...that are on plasmid pXO1 and pXO2. Due to the plasmid can be affected by environmental impact, many authors have established the specific primers which located in chromosome are stable than in plasmid.

In this study, we used the primers which reported by WHO and National Institute of Infectious Disease, Tokyo, Japan (table 1). The following PCR cycle was used: 1x 94^0C for 5min; 30 x (94^0C for 30s followed by 55^0C for 30 s and 72^0C for 55s); 1 x 72^0C for 5 min; cool to 4^0C.

PCR results indicated that the PCRs method could be directly detected the virulent genes of *B.anthracis* from isolate strains, clinical samples and environmental samples in Vietnam. The results also showed the advantage of PCR method because it has determined *B.anthracis* virulence genes from the clinical samples while culture result was negative. In addition, the PCR result can be analyzed by multiple-locus variable number tardem repeat to describe molecular characterization of obtained strains and compared with *B.anthracis* strains in different countries.

As several studies reported, it is very difficult to directly detect anthrax DNA from soil samples. In this study period, moreover, PCR results of the extracted DNA from 70 soil samples were negative with *B.anthracis*. Then, we made an amount of anthrax spore from 34F2DK *B.anthracis* strain and mixed with soil sample (which was negative by culture and PCR). Results indicated that anthrax DNA from soil was detected by PCR using Ba813 R1/R2 primers. However, the result was based on the experimental stage because the spores were created from *B.anthracis* strain. In addition, we have to identify the concentration of spore for PCR reaction and also detect anthrax DNA directly from a real soil sample. It could be done in the next period. Therefore, we plan to collect more samples in the selected study areas and also find out a linked between animal – human- environment by using modern molecular methods.

Another result of the present study is the Anthrax network has successful created in Vietnam, especially in the high risk areas in the North of Vietnam. We have the anthrax teams in the preventive medicine center in the provinces and others in the communes. They are available to contact by the hot line and/or cell phone and make a monthly report of anthrax to NIHE.

6.11 Publications

None

6.12 Reference

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6.13 Acknowledgements

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Sincerely, thank to Department of International Cooperation, NIHE for administration support.

Research 7

7.1. Project title: ***Enhancement of the National Institute of Hygiene and Epidemiology rabies laboratory capacity for rabies/bat lyssavirus diagnosis and research***

7.2. General objectives: To enhance National Institute of Hygiene and Epidemiology rabies laboratory capacity for rabies/bat *lyssavirus* diagnosis and research.

7.3. Specific Objectives

- ***Objectives for the first year:*** To establish and evaluate the FAVN technique for bat *Lyssavirus* antibody detection.
- ***Objectives for the second year:*** To produce purified and recombinant viral protein of rabies/bat *lyssaviruses* for ELISA technique and for production of other biological materials.
- ***Objectives for the third year:*** To establish and evaluate the ELISA test to detect rabies/bat *lyssavirus* antibodies.

7.4. Name of Researchers

- Dr. Nguyen Thi Kieu Anh, Dr. Nguyen Vinh Dong, Dr. Nguyen Tuyet Thu, Dr. Ngo Chau Giang, Dr. Nguyen Thi Hong Hanh and Dr. Nguyen Tran Hien (National Institute of Hygiene and Epidemiology, Vietnam).
- Dr. Satoshi Inoue, Dr. Akira Noguchi (National Institute of Infectious Diseases, Japan).

7.5. Affiliation

- National Institute of Hygiene and Epidemiology, Vietnam.
- National Institute of Infectious Diseases, Japan.

7.6. Sub-project title: Establishment and evaluation of the FAVN technique for bat *Lyssavirus* antibody detection.

7.7. Summary

Although, Vietnam has been in the high epidemic region of rabies with over 300 human rabies cases were reported and rabies epidemic occurred in 27 provinces from 2007 – 2010. But to date, a national program for rabies control and prevention has not been established. Therefore, to improve surveillance and control of rabies, it is important to establish a national program for rabies prevention and control including intervention and cooperation between animal and human health combined with laboratory network for rabies diagnosis. The objective of project was to enhance the NIHE rabies laboratory capacity for rabies/bat *lyssavirus* diagnosis and research. In the first year of project, we aimed to establish and evaluate the FAVN technique for bat *Lyssavirus* antibody detection. The establishment of FAVN for screening bat sera which contained the neutralization antibodies against *lyssaviruses,* and FAVN for titration neutralization antibodies against *lyssavirus* in bat sera, using 5 rabies/*lyssaviruses* were developed by NIHE and NIID. Training technique for NIHE staff was also done in the collaboration with NIID laboratory. One staff of NIHE was sent to NIID to learn by performing 260 bat serum samples under supervisor of NIID researcher. To evaluate the proficiency testing of NIHE laboratory, the same SOPs of FAVN were used to test concurrently one hundred of bat sera by both NIID and NIHE laboratories. The results showed that NIHE staff can perform FAVN with 99 – 100% of correlative test results with NIID, depending on the virus strain use. In addition, the prototype results of sero surveillance of bat *lyssaviruses* proved that the evidence of neutralization antibodies against CVS and EBLV 1 in bat sera in Northern Vietnam with 30/100 and 34/100 respectively. In conclusion, SOPs of FAVN technique for detection of neutralization antibody against 5 bat *lyssaviruses* were developed and will be applied in NIHE and other institutions for sero surveillance of rabies and bat *lyssaviruses* in Vietnam.

7.8. Purposes

The genus *Lyssaviruses* are recognized in the family *Rhabdoviridea* and were reported to cause rabies or rabies like diseases throughout the world. To date, *Lyssaviruses* that cause human diseases classified into 7 genotypes, namely genotype 1, Rabies virus (RABV); 2, Lagos bat virus (LBV); 3, Mokola virus (MOKV); 4, Duvenhage virus (DUVV); 5, European bat *lyssavirus* type 1 (EBLV-1); and 6, EBLV-2, and finally, Australian bat *lyssavirus* (ABLV). Phylogenetic analyses of the seven genotypes and their pathogenic and immunogenic properties enabled further division into two major phylo-groups [1]. Phylogroup I includes RABV, the classic rabies virus that is present worldwide, as well as DUVV, EBLV-1, EBLV-2, and Australian Bat Lyssa virus that related to two human deaths (ABLV), while phylogroup II comprises MOKV and LBV. The cross neutralizing antibody against the viruses in the phylo-groups were demonstrated [3,4].

In Vietnam as well as in other Asian countries only rabies virus (RABV), genotype 1 of the *Lyssaviruses* that causes human rabies has been recognized and reported with the main reservoirs are dogs and cats [7]. Some research on *Lyssaviruses* in bat reservoirs had been carried out in several South -East countries such as the Philippines, Cambodia and Thailand proved that bat *Lyssaviruses* are naturally circulating in both insectivorous and frugivorous bats [2,5,6]. In Vietnam, the research on other members of the *Lyssaviruses* both in animal reservoirs and in humans has rarely been carried out. From 1996 – 2005, a total of 1,038 human rabies deaths reported by the program for human rabies control and prevention. Among them 88% and 9% had dog and cat bites respectively. Interestingly, 2% had contacted with dogs or cats during the butchering process and 1% had exposure to other animals such as monkey, mouse and unknown [8]. Furthermore, according to the results obtained by our research group that were conducted from 2006-2009, out of 31 patients who admitted to the hospitals with the rabies like symptoms, only 23 patients were confirmed to be infected with RABV. Evidence of RABV infection could not be found by either by RT-PCR or by serological detection of anti-RABV antibody in the sera taken from the rest of those patients. Among the patients who were confirmed to be infected to rabies virus, 6 people (26%) did not have any history of dog/cat bites or butchering sick animals. This raises a question as to whether other genotypes of the *Lyssaviruses* might be circulating in Vietnam.

In recent years, Vietnam as well as the Philippines, Laos, Cambodia, and China are facing the problem of rapidly increasing human rabies cases. From 2007 to 2010 over 300 human rabies cases were reported and rabies epidemic occurred in 27 provinces in Vietnam [8]. Although, Vietnam is in the high epidemic region of rabies, but to date, a national program for rabies control and prevention has not been established; only a system for human rabies surveillance, control and prevention is available and belongs to Ministry of Health. The annual data of human deaths due to rabies reported by this system is based only on the clinical diagnosis, not by laboratory confirmation. Furthermore, the symptoms of rabies are not specific in the early period of the illness, so it may be confused with other encephalitic diseases. This is why rabies confirmation in the early stages is very important and requires isolation of the patient to prevent potential exposure to the rabies virus of the patient's relatives and health care workers, and with the laboratory confirmation, the data of rabies surveillance in both human and animals will be more precisely. Therefore, to improve surveillance and control of rabies, it is important to establish a national program for rabies prevention and control including intervention and cooperation between animal and human health combined with laboratory network for rabies diagnosis.

From 2007 – 2009, the rabies laboratory at National Institute of Hygiene and Epidemiology, Hanoi (NIHE) had been supported by Ministry of Health to implement the Ministry level project ***"Research on rabies virus molecular epidemiology in Vietnam"*** and from 2008- 2010, it was supported by National Institute of Infectious Diseases, Japan (NIID) to carry out the project titled ***"Development, application of new methods for rabies diagnosis in NIHE"***. From these projects, three regional laboratories in three provincial preventive medicine centers were set up for animal and human sample collection. They are located in the north (Ha Tay province), highland (Gia Lai province) and the center of Vietnam (Binh Thuan province). Three laboratories were equipped with the essential tools, facilities for animal operation, and all staff of those laboratories were vaccinated and trained on bio-safety, techniques for operating animals, taking, packing and shipping samples. Since 2008, those laboratories have started operations to take samples of rabies suspected animals in the regions. By now, 176 dog and 50 human samples have been collected and analyzed. To step by step set up the net work for rabies diagnosis, it is essential to enhance the rabies diagnosis/research capacity for laboratories at

National level (National Institute of Hygiene and Epidemiology), then extend to the lower levels of both animal and human health diagnosis centers.

The objective of first year project is to establish and evaluate the FAVN technique for bat *Lyssavirus* antibody detection.

7.9 Methods

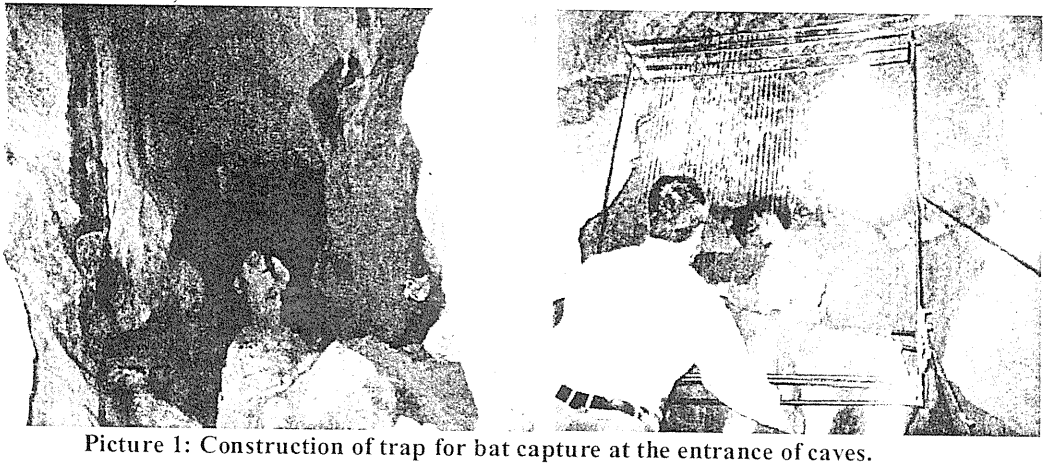
7.9.1 Materials

- 360 bat serum samples collected in 6 Northern provinces of Vietnam.
- 5 strains of *Lyssaviruses:* Rabies virus (RABV); Lagos bat virus (LBV); Mokola virus (MOKV); 4, Duvenhage virus (DUVV); 5, European bat *lyssavirus* type 1 (EBLV-1) were kindly supported by NIID and National Center for Scientific Research (NCSR), France.

7.9.2 Methods

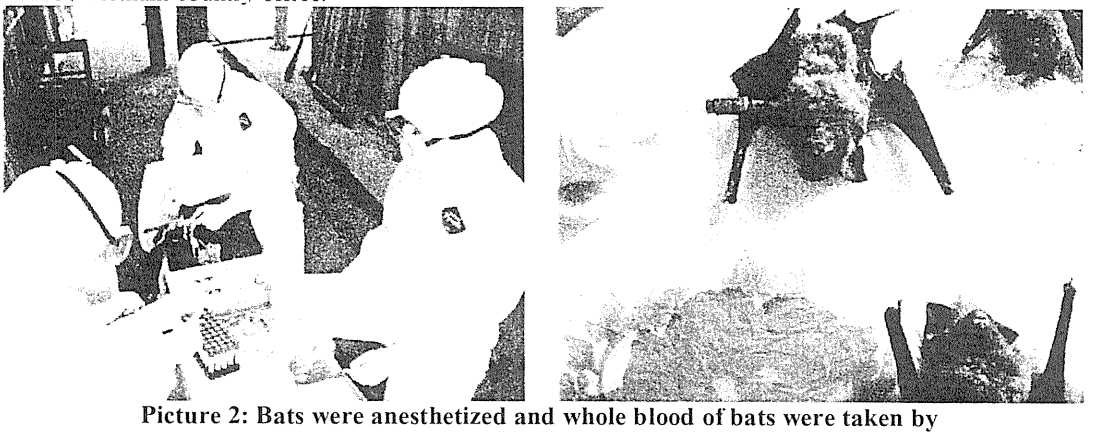
7.9.2.1 Sample collection and identification.

Bats were captured in 6 Northern provinces (Tuyen Quang, Phu Tho, Yen Bai, Hoa Binh, Lang Son and Bac Giang) by mist nest or trap (Picture 1). Then, captured bats were transported to the assembly points in the special cages (having the small holes for bat hanging; being moisture, fresh environment and dark).



Picture 1: Construction of trap for bat capture at the entrance of caves.

At the assembly points, bats were anesthetized by ketamine hydrochloride and whole blood of bats were taken by heart puncture (picture 2). The bat sera were separated by centrifugation and coded, then transferred together with bat bodies under cold change to NIHE for conservation and further analyses. At NIHE, the bats were initially characterized by the expert of Ecology and Biological Resources Institute by appearance (wing, ear, face, skull cap/jaw... characteristics), then by echo and or molecular methods if it is necessary. Bat collection and identification were financially supported by WHO, Vietnam country office.



Picture 2: Bats were anesthetized and whole blood of bats were taken by heart puncture

7.9.2.2 Receiving and developing the master seed virus (MSV)/working seed virus (WSV) of 5 Lyssaviruses strains.

CVS strain was kindly supported by the Center for Viral Structure Research, National Center for Scientific Research, France in 2005 and was produced MSV, WSV for FAVN test.

Four other *lyssavirus* trains (Lagos bat virus (LBV); Mokola virus (MOKV); Duvenhage virus (DUVV); European bat *lyssavirus* type 1 (EBLV-1) were transferred to NIHE by NIID after the agreement of biological transfer was signed by directors of NIHE and NIID as well as agreement of biological material importation was signed by General Department of Preventive Medicine, Ministry of Health, Vietnam. The production of MSV and WSV of all four *lyssaviruses* strains were implemented in NIHE laboratory by propagating on NA cell line, following the SOPs of NIHE.

7.9.2.3 Developing the FAVN technique.

The detail SOP of FAVN for screening bat sera which contained the neutralization antibodies against *lyssaviruses,* and FAVN for titration neutralization antibodies against *lyssavirus* in bat sera, using 5 rabies/*lyssaviruses* were developed by NIHE and NIID.

Training technique for NIHE staff was also done in the collaboration with NIID laboratory. One staff of NIHE was sent to NIID to learn by performing 260 bat serum samples under supervisor of NIID researcher.

The same SOPs were used to test concurrently one hundred of bat sera by both NIID and NIHE laboratories. The results were compared to evaluate the proficiency testing of NIHE laboratory.

7.10 Results

7.10. 1. Bat species collected in 6 Northern provinces.

A total of 926 bats were collected, identified and classified into 25 species of insectivorous bats and 2 species of frugivorous bats (table 1). 578 bat sera were collected, but only 260 bat sera that had volume over 300 µl were chosen to be used for proficiency testing and inter (NIID – NIHE) laboratory comparison.

Table 1: Bat species and number of bat/each species were captured in 6 provinces

Identity	Bat species	Number of samples
Insectivorous	Taphozous theobaldi	74
	Tadarida plicata	32
	Taphozous cf. melanopogon	223
	Taphozous sp	25
	Pipistrellus sp	19
	Hipposiderous larratus	133
	Rhinolophus macrotis (small)	16
	Miniopterus sp	13
	Rhinolophus macrotis (large)	18
	Myotis sp2	11
	Aselliscus stoliczknus	23
	Hypsugo sp2	17
	Ia io	46
	Myotis sp1	13
	Rhinolophus affinis	11
	Hypsugo sp1	11
	Rhinolophus cf pearsoni	21
	Rhinolophus cf pusillus	9
	Aselliscus stoliczkanus	13
	Hipposideros alongensis sungi	19
	Rhinolophus cf. microglobossus	40
	Aselliscus stoliczkanus	7
	Hiposideros armiger	11
	Rhinolophus pusillus	24
	Miniopterus sp. Cf. schreibersi	27

Frugivorous	Rousettus sp	16
	Eonycteris spelaea	9
	Total	**926**

7.10. 2. Detail SOP of FAVN

7.10.2.1 SOP of FAVN for screening of bat sera contained neutralization antibodies against lyssaviruses.

❖ **Propagation of virus for neutralization test**. This work is done in the P3 laboratory.

Cell growth: the NA cell line used to produce the CVS, Mokola, EBLV1, Duvenhage, Lagos viruses are trypsinised during the rapid growth phase. Cells are suspended within a volume of 20–30 ml in cell culture medium with 10% heat-inactivated FCS.

Infection of cells: the multiplicity of infection (number of infective particles per cell) is adjusted to between 0.1 and 0.5. The glass bottle containing the virus/cell suspension is incubated for 60 minutes at 36°C ± 1°C. The bottle are gently stirred every 10–15 minutes during the incubation time.

Virus growth: the virus/cell suspension is then centrifuged at 800–1000 ***g*** for 15 minutes and the cell pellet is resuspended in cell culture medium mixed with 10% heat-inactivated FCS. Total of $2x10^7$ cells in 30ml of grow medium (DMEM 10%FCS) are cultured in the $75cm^2$. Concurrently, 100 µl of infected cell suspension is added into each well of chamber slide. Two days for CVS, Mokola, EBLV1, Lagos strains and three days for Duvenhage strain after post inoculation, the chamber slides are fixed by 80% acetone and stained with 50 µl of DFA(Fujirebio) at the concentration of 40 fold dilution by PBS (-) included 0.002% Evan's Blue for determination of infected cell percentage. If 100% of cell infected, the virus should be harvested.

Harvest and storage: the supernatant is centrifuged at 800–1000g for 15 minutes at 4°C. If several flasks have been used, the different centrifuged supernatants are mixed and then aliquoted and frozen at –80°C.

❖ **Titration of virus in $TCID_{50}$ (50% tissue culture infective dose)**

This titration method uses NA cell line in microtitre plates. Cell inoculation and infection experiments are carried out in BSL3 laboratory.

Dilution of the virus: the serial dilutions are performed in U microplate 30µl virus in 270µl cell culture medium without FCS as diluent. Tenfold dilutions from 10^{-1} to 10^{-7} are prepared. 50 µl of each virus suspension is transferred to each well of microplate, six replicates are used per dilution.

Cell suspension: A flask of confluent cell is trypsinized then a cell suspension containing $8x10^5$ cells/ml is prepared in cell culture medium containing 10% heat-inactivated FCS, and is distributed 50 µl per well, into 96-well microtitre plates contained virus. The plates are then incubated 48 hours at 36°C ± 1°C with 5% CO_2.

Incubation: incubate for 48 hours at 36°C ± 1°C in 5% CO_2

Staining and calculation of titre: The cells are then washed with PBS three times, fixed with 200 µl/well of 80% cold aceton and stained with DFA(Fujirebio) 35µl/well at the concentration of 1/40 in DW, incubation for 30min at RT. Reading is qualitative (all or nothing – ON – OFF) every well that shows specific fluorescence is considered to be positive. The titre calculation is made using the Spearman–Kärber formula.The infective dose uses in FAVN is 100 $TCID_{50}$/50 µl.

(The CVS titration must be performed by FAVN test to establish the infective dose in $TCID_{50}$).

Neutralization test

+ Heat inactivated serum at 56°C/30 minutes
+ Serum dilution: 1/5 (10 µl serum + 40 µl of diluent (DMEM)
+ Virus (100$TCID_{50}$/50µl): 50 µl
+ Incubation: 36 ± 1°C /1hr, CO_2 incubator
+ Add MNA cells (8×10^5/ml): 50 µl (fresh preparation)

+ Incubation: 36°C ± 1°C/ 48hrs
+ Remove medium(by micropipette tip)
+ Add PBS- (Rinse): 150µl/well
+ Remove PBS-(by micropipette tip)
+ Dry up:1 – 2 hr(s) at RT
+ Cold 80% acetone fix: 200 µl /well, 4°C for 30min
+ Remove acetone(decantation), PBS wash once, 150µl/well
+ DFA(Fujirebio): 35µl/well at the concentration of 1/40 in DW, incubation for 30min at RT.
+ Wash (PBS-): 3times (200µl)
+ Observation by microscope (UV)(10x 10).

Interpreting results: At least a 90% reduction in infectious centers was observed relative to the positive control. All positive samples were retested at increasing dilutions to estimate endpoint antibody titers in NIHE [6, 9].

7.10.2.2 SOP of FAVN for titration of neutralization antibodies against lyssaviruses in bat sera.

The SOPs for virus propagation and titration as the same as SOPs for screening of bat sera contained neutralization antibodies against *lyssaviruses*.

Neutralization test

+ Heat inactivated serum at 56°C/30 minutes.
+ Serum testing and standard serum dilution (2IU): serum testing as well as standard serum are diluted in the microplate. Six replicates per each dilution should be performed.

	x5	x10	x20	x 40	x80	x 160	
Serum	20µl	50µl	50µl		. . . . .		50µl
Diluent	80 µl	50µl	50µl				

+ Add challenge virus (100$TCID_{50}$/50ul): 50 µl of working challenge virus is added per well. Challenge virus back titration should be performed in 10 fold serial dilution from 10^{-1} – 10^{-4}, then 50µl of each dilution is added per well, 6 replicates should be done per each dilution.
+ Incubation: 36 ± 1°C /1hr, CO_2 incubator
+ Add cells: MNA(8 x 10^5/ml): 50 µl (fresh prepare)
+ Incubation: 36°C ± 1°C/ 48hrs
+ Remove medium(by micropipette tip)
+ Add PBS- (Rinse):150µl/well
+ Remove PBS-(by micropipette tip)
+ Dry up:1 – 2 hr(s) at RT
+ Cold 80% acetone fix: 200 µl /well, 4°C for 30min
+ Remove acetone(decantation), PBS wash once, 150µl/well
+ DFA(Fujirebio): 35µl/well at the concentration of 1/40 in DW, incubation for 30min at RT.
+ Wash (PBS-): 3times (200µl)
+ Observation by microscope (UV)(4 x 10).

Reading is qualitative (all or nothing – ON – OFF) every well that shows specific fluorescence is considered to be positive. The 50% end point neutralizing titers were calculated by the method of Reed and Muench . Only the samples that had a 50% end point neutralizing titer greater than $1\log_{10}$ is considered as positive [9,10].

7.10. 3. Results of proficiency testing/inter laboratory comparison.

7.10.3. 1. The proficiency testing/inter laboratory comparison of FAVN, using CVS strain

Table 2: The FAVN test results to determine the neutralization antibody against CVS strains of bat sera obtained by NIID and NIHE

FAVN performed by NIID	FAVN performed by NIHE	
	Positive	Negative
Positive	30	1
Negative	0	69

Using CVS strain as challenge virus to perform FAVN test to detect neutralization antibody against CVS in 100 bat sera, we found that the correlative test results obtained by NIID and NIHE were 99/100 samples, only one sample considered as positive by NIID but negative by NIHE. At least 30% of bat sera among 100 bats were positive with antibody against CVS strain.

7.10.3. 2. The proficiency testing/inter laboratory comparison of FAVN, using EBLV1 strain.

Table 3: The FAVN test results to determine the neutralization antibody against EBLV1 strain of bat sera obtained by NIID and NIHE.

FAVN performed by NIID	FAVN performed by NIHE	
	Positive	Negative
Positive	34	0
Negative	0	66

Neutralization of EBLV1 virus strain, we found that 34% of bat sera were positive with antibody against EBLV1 *Lyssavirus* strain. The correlative test results obtained by NIID and NIHE were 100%.

7.10.3. 3. The proficiency testing/inter laboratory comparison of FAVN, using Lagos strain

Table 4: The FAVN test results to determine the neutralization antibody against Lagos strain of bat sera obtained by NIID and NIHE.

FAVN performed by NIID	FAVN performed by NIHE	
	Positive	Negative
Positive	0	0
Negative	0	100

The results presented in table 4 showed that no evidence of neutralization antibody against Lagos virus among 100 bat sera. The correlative test results obtained by NIID and NIHE were 100%.

7.10.3. 4. The proficiency testing/inter laboratory comparison of FAVN, using Mokola strain

Table 5: The FAVN test results to determine the neutralization antibody against Mokola strain of bat sera obtained by NIID and NIHE.

FAVN performed by NIID	FAVN performed by NIHE	
	Positive	Negative
Positive	0	0
Negative	0	100

The result of FAVN test to detect neutralization antibody against Mokola virus showed that, no serological evidence of Mokola virus infection among 100 bats collected in 6 Northern provinces, Vietnam. The correlative test results of FAVN test were 100% in inter NIHE and NIID laboratory comparison.

7.9. Discussions

Rapid Fluorescence Focus Inhibition Test (RFFIT) is considered as the gold standard to detect and titrate the neutralization antibody against rabies/*lyssaviruses*. But, to perform this test with large number of samples, it is really a big challenge to the laboratory workers due to time as well as hard working on observing and counting fluorescence focus under x 200 magnification of fluorescence microscope. So, modified RFFIT, an easier and effective method, the Fluorescence Antibody – Virus neutralization (FAVN) test was developed and evaluated by several authors to apply on detection of neutralization antibody in immunized human, animals as well as reservoirs. The absolutely correlative test results of FAVN and RFFIT were demonstrated. Therefore, OIE allowed using FAVN to determine antibody level against rabies in sero surveillance of dogs or wildlife [10]. Under collaboration of NIHE and NIID, we established the FAVN technique, using different strains of bat *lyssaviruses* to screen and titrate neutralization antibodies against bat *lyssavirus* in order to conduct the research to identify bat

lyssaviruses in Vietnam as well as to apply on sero surveillance of rabies neutralization antibody in domestic animals for immunization campaign and rabies control and prevention. Furthermore, in this collaborative research, NIHE was inherited four specious strains of bat *lyssaviruses* from NIID, these viruses will be the reference strains for continued researches on bat *lyssaviruses*, that help to strengthen research capacity of NIHE as well as help to improve the PMS on prevention and control of rabies/*lyssaviruses* in the future .

The proficiency testing/inter NIHE and NIID laboratory comparison of FAVN, using **CVS strain** was done independently among two laboratories by using the same 100 samples of bat sera. We found that one bat serum was negative by NIHE experiment, while positive by NIID experiment. This may be the results of: (i) infectivity of the CVS virus challenge ($100TCID_{50}$) in the experiments were not totally the same ; (ii) the titer of neutralization antibody in that bat serum was low; (iii) the passage level of CVS and NA cell were not identical in the experiments, so that led the propagation ability of virus used in the experiments were not completely identical; (iv) the reading result was based on at least 90% of reduction in infectious centers was observed relative to the positive control, so the results may be depended on the skill and experiences of laboratory staff; (v) this FAVN for screening of neutralization antibody is semi quantitative method, so the results may be also influenced by the quality of the equipments such as microscopy, pipettes, tips... Therefore, it is important to calibrate the equipments regularly to ensure the quality of the experiments. However, it is necessary to confirm the neutralization antibody contained in this bat serum, the titration of neutralization antibody titer should be performed by using 50% endpoint of inhibition compared with the standard serum.
In addition, we found the evidences of bat *lyssaviruses* circulating in Northern Vietnam. These findings will lead up to research on rabies related diseases in unknown encephalitis patients. The findings of this research will help the policymakers to establish policies on biological resource preservative program and develop safety touring services in the regions or caves where bat *lyssaviruses* are circulating as well as to aware the risk of bat *lyssavirus* infection to the public health concerning to the bat eating habit and using of bat guano for fertilizer purpose or other issues.

7.10. Conclusions

SOPs of FAVN technique for detection of neutralization antibody against 5 bat *lyssaviruses* were developed and applied in NIHE for sero surveillance of bat *lyssaviruses* in Vietnam. The proficiency testing and inter laboratory comparison showed that NIHE can perform FAVN test with 99 – 100% of correlative test results with NIID, depending on the virus strain use.

The prototype results of sero surveillance of bat *lyssaviruses* proved that the evidence of neutralization antibodies against CVS and EBLV 1 in bat sera in Northern Vietnam with 30/100 and 34/100 respectively.

7.11. Publications

The prototype result of serological evidence of bat *lyssaviruses* in 6 northern provinces, Vietnam was accepted and presented at the second international conference on zoonotic diseases which was held in Taiwan by Animal Health Research Institute, Council of Agriculture, Executive Yuan, from 5 – 9, December, 2011.

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7.13. Acknowledgements

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Research 8

8.1 Project title: ***Phylogenetic analysis and transmission dynamics of measles and rubella viruses isolated from some outbreaks in the Northern provinces of Vietnam from 2006 to 2014.***

8.2 General objectives:

a. Identify incidence and trends of measles/rubella
b. Describe epidemiological characteristics measles/rubella
c. Identify genotypes, subgenotypes and genetic characteristics of measles and rubella viruses circulated in the Northern provinces of Viet Nam from 2006 – 2014.

8.3 Specific Objectives

a. Identify genotypes and genetic characteristics of measles and rubella viruses circulated in the Northern provinces of Viet Nam from 2006 – 2009.

8.4 Name of Researchers

a. National Institute of Hygiene and Epidemiology, Vietnam: Trieu Thi Thanh Van, MSc; Nguyen Phan Le Anh, M.D, M.P.H., PhD.
b. National Institute of Infectious Diseases, Japan: Dr. Katsuhiro Komase, Department of Virology III

8.5 Affiliation

a. National Institute of Hygiene and Epidemiology, Vietnam.
b. National Institute of Infectious Diseases, Japan.

8.6 Sub-project title: Phylogenetic analysis of measles and rubella viruses isolated from some outbreaks in the Northern provinces of Vietnam from 2006 to 2009.

8.7 Summary

This study will be based on the national epidemiological and laboratory routine surveillance of measles and rubella infection. It is estimated that about 300 throat swabs samples will be collected from patients with rash, fever. Epidemiological, virological, clinical and vaccination information of patients will also be collected by interviewing commune / village health staff, patient's parent, by reviewing hospital records, case investigation forms, outbreak reports and by checking immunization cards and log books. Genetic characterization of wild-type measles virus (MV) was studied using nucleotide sequencing of the C-terminal region of the N protein gene and phylogenetic analysis on 49 isolates. Genetic characterization of wild-type rubella virus (RV) was studied using 739 nucleotide sequencing (nucleotides 8,731-9,469) of E1 glycoprotein and phylogenetic analysis on 14 isolates from provinces in the North Vietnam in 2008-2009. The results showed that all of the strains of MV belonged to genotype H1. 6 MV isolates in 2006 were belonged to cluster 1 and 43 isolates in 2008-2009 were cluster 2. The nucleotide sequence homologies of the 49 H1 strains were 97.8%–100%. 14 RV isolates in 2008-2009 were belonged to genotype 2B. The nucleotide sequence homologies of the 14 2B strains were 98.6%–100%. The report showed that the transmission of genotype H1 of MV and genotype 2B of RV in the North Vietnam in 2006-2009.

8.8 Purposes

Measles and rubella are similar rash illnesses that may be difficult to differentiate clinically. The routine procedure, laboratory confirmation of suspected cases is based on detection of virus specific immunoglobulin M (IgM) in a single blood after rash onset, molecular techniques such as reverse-transcription polymerase chain reaction (RT-PCR) to detect viral RNA are often used to complement serologic testing. An important aspect of laboratory surveillance for measles and rubella is the genetic characterization of circulating wild-type viruses to support molecular epidemiologic studies. These studies can help to measure transmission pathways and to clarify epidemiological links during outbreaks. Virological surveillance that is sufficient to document the interruption of transmission of measles and rubella viruses will be an essential criterion for verification of elimination.

Measles virus (MeV) is a single-stranded, negative-sense RNA virus, belonging to the genus *Morbillivirus*, family *Paramyxoviridae*. Measles is a vaccine-preventable disease, but is still a major killer of infants worldwide. During 2000-2008, global measles mortality declined by 78%, from an estimated 733,000 deaths in 2000 to 164,000 in 2008. The genome consists of 15,894 nucleotides, which code for the six structural proteins, the nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H), and large protein (L), and two nonstructural proteins, C and V. The nucleotide sequences of the L, M, and F genes are much less variable than the sequences of the N, P, and H genes, which have 7%–10% variability. The N and H gene sequences are most commonly used for genetic characterization of wild-type viruses. In particular, one of the most variable parts of the measles genome is the 450-nucleotide region, which codes for the COOH terminal 150 amino acids the N protein, where nucleotide variability can approach 12% between wild-type viruses.

Rubella virus (RV) infection is usually mild or asymptomatic in children and adults. The estimated annual incidence of CRS cases worldwide was 100,000 in 2003. RV is the sole member of the genus *Rubivirus*, in the family *Togaviridae*. The virus has a single-strand, positive sense RNA genome of 9,762 nucleotides (nt) that encodes 2 nonstructural polypeptides (p150 and p90) within its 5_-terminal two-thirds and 3 structural polypeptides (C, E2, and E1) within its 3_-terminal one-third. The E1 glycoprotein is considered immunodominant in the humoral response induced against the structural proteins and contains neutralizing and hemagglutinating determinants. A 739-nt region within the E1 gene (nt 8731 to 9469) is accepted as the minimum amount of sequence information required for molecular epidemiological purposes. Nine rubella virus genotypes (1B, 1C, 1D, 1E, 1F, 1G, 2A, 2B, and 2C) and 4 provisional genotypes (1a, 1 h, 1i, and 1j) based on sequence variation in the 739-nt region have been established.

8.9 Methods.

a. ***Specimen Collection:*** Staff members from the Provincial centre for Preventive Medicine collected throat swab specimens from patients during the outbreak (from 28 provinces in the North Vietnam and from 2006 to 2009). Throat swab specimens were obtained according to the WHO procedures for laboratory diagnoses of measles and rubella virus infections and transported to the National Measles Laboratory, National Institute of Hygiene and Epidemiology for processing by standard procedures. To confirm all suspected cases, we used ELISA kits (Siemens, Germany) to detect measles and rubella virus IgM.

b. ***Virus Isolation, PCR, and Sequencing:*** Measles virus or rubella virus was isolated by using the Vero/hSLAM cell line. The Vero/hSLAM cell line is now recommended for routine isolation of measles and rubella viruses in the WHO laboratory network. These cells are Vero cells that have been transfected with a plasmid encoding the gene for the human signaling lymphocyte activation molecule (hSLAM) protein. SLAM has been shown to be a receptor for wild type of measles and measles infection of Vero/hSLAM cells results in the characteristic cytopathic effect (CPE). Rubella virus (RV) from clinical samples grows similarly in both Vero and Vero/hSLAM cells but do not cause a reproducible CPE in either. The infected cells were harvested when more than 75% of the culture showed CPE. RNA was extracted from supernatant by using the QIAamp Viral RNA Mini Kit (QIAGEN) according to manufacturer's instructions. Reverse transcription–PCR (RT-PCR) was used to amplify the 634 nt coding for the COOH terminus of the N gene. Meanwhile, RNA was extracted from supernatant without CPE. Reverse transcription–PCR (RT-PCR) was used to amplify either the 185 nt coding for the partial E1 gene to detected rubella virus and then RT-PCR was used to amplify fragment 1 (480 nt), fragment 2 (633 nt) coding for the E1 gene of rubella virus. PCR products were purified by using a QIAquick PCR Purification Kit (QIAGEN). Sequences of the amplicons were obtained by using BigDye terminator version 2.0 chemistry according to the manufacturer's protocol for both sense and antisense strands on an automated 3100 Avant DNA Sequencer (Applied Biosystems). Phylogenetic analyses were performed and trees were generated by using MEGA4 (www.megasoftware.net). The robustness of the groupings was assessed by using bootstrap resampling of 1,000 replicates.

c. ***Case investigation:*** Suspected measles / rubella cases (SMR cases) who onset dated 1/1/2006 to 31/12/2009 were investigated with case investigation forms and their throat swabs samples were taken. Case investigation form includes information of personal information, address, date of rash, contact history, immunization history, symptoms, complications, sample taken date. Study sites were any places in the Northern provinces where outbreaks occurred. From 2006 – 2009, outbreaks happened in 13 provinces including Dien Bien (2006), Thai Nguyen (2006), Lai Chau (2006), Thanh Hoa, Ninh Binh,

Ha Noi, Bac Ninh, Hai Duong, Hoa Binh, Nghe An, Thai Binh, Bac Giang and Ha Nam. However, due to time-lag between date of onset and date of research, some information were missing in few cases.

8.10 Results

In 2006, 18 throat swab specimens was collected from patients with rash and fever during the outbreak in three provinces: Dien Bien, Thai Nguyen and Lai Chau. There is no throat swab sample taken in 2007 because of no report of outbreaks. In 2008, 42 thoat swabs were collected from patients during the outbreak in three provinces: Ninh Bình (May and June), Thanh Hoa (November) and Ha Noi (November and December). In 2009, 97 thoat swab specimens were collected from patients during the outbreak in 9 provinces: Ha Noi, Ninh Binh, Bac Ninh, Hai Duong, Hoa Binh, Nghe An, Thai Binh, Bac Giang and Ha Nam.

In 2006, 6 measles virus isolates were obtained from 18 throat swab specimens: 1 from mountainous Sin Ho district, Lai Chau province, 2 from mountainous Dien Bien City, Dien Bien and 3 from hilly Thai Nguyen city, Thai Nguyen.

Table 1: Description of measles viruses in 2006

Patient No.	Patient Age	Address		Result		Strain name
		District	Province	Virus isolation	RT -PCR	
1	2	Sin Ho	Lai Chau	+	+	MVi/LaiChau.VNM/6.2006/1
2	10	Tua Chua	Dien Bien	+	+	MVi/DienBien.VNM/7.2006/4
3	7	Tua Chua	Dien Bien	+	+	MVi/DienBien.VNM/7.2006/5
4	16	Thai Nguyen	Thai Nguyen	+	+	MVi/ThaiNguyen.VNM/15.2006/15
5	16	Thai Nguyen	Thai Nguyen	+	+	MVi/ThaiNguyen.VNM/15.2006/16
6	16	Thai Nguyen	Thai Nguyen	+	+	MVi/ThaiNguyen.VNM/15.2006/18

In 2008, 17 measles virus isolates were obtained from 42 throat swab specimens: 6 from Nho Quan disitrict, Ninh Binh province, 3 from Sam Son district, Thanh Hoa and 8 from Ha Noi, they located in Dong Da district (1), Hai Ba Trung (1), Ha Dong (2) and 4 unknown location. Meanwhile, 2 rubella virus isolates from Nho Quan disitrict, Ninh Binh province.

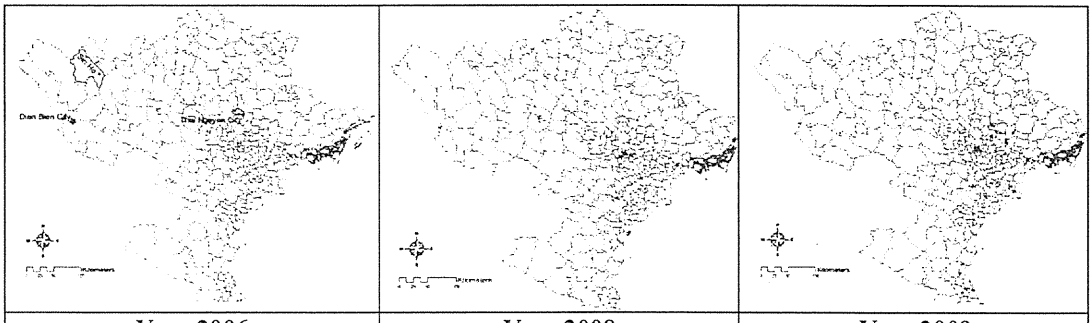
Table 2: Description of measles and rubella viruses in 2008

Patient No.	Patient Age	Address		Result		Strain name
		District	Province	Virus isolation	RT - PCR	
7	18	Nho Quan	Ninh Binh	+	+	MVi/NinhBinh.VNM/20.2008/3
8	18	Nho Quan	Ninh Binh	+	+	MVi/NinhBinh.VNM/19.2008/4
9	18	Nho Quan	Ninh Binh	+	+	MVi/NinhBinh.VNM/20.2008/5
10	38	Nho Quan	Ninh Binh	+	+	MVi/NinhBinh.VNM/20.2008/6
11	20	Nho Quan	Ninh Binh	+	+	MVi/NinhBinh.VNM/23.2008/8
12	14m	Nho Quan	Ninh Binh	+	+	MVi/NinhBinh.VNM/23.2008/10
13	7	Ha Dong	Ha Noi	+	+	Mvi/HaNoi.VNM/47.2008/12
14	18	Ha Dong	Ha Noi	+	+	Mvi/HaNoi.VNM/47.2008/15
15	16		Ha Noi	+	+	Mvi/HaNoi.VNM/47.2008/16
16	1	Sam Son	Thanh Hoa	+	+	Mvi/ThanhHoa.VNM/48.2008/18
17	4	Sam Son	Thanh Hoa	+	+	Mvi/ThanhHoa.VNM/49.2008/19
18	5	Sam Son	Thanh Hoa	+	+	Mvi/ThanhHoa.VNM/49.2008/21
19	20		Ha Noi	+	+	Mvi/HaNoi.VNM/50.2008/25
20	24	Dong Da	Ha Noi	+	+	Mvi/HaNoi.VNM/50.2008/28
21	24		Ha Noi	+	+	Mvi/HaNoi.VNM/50.2008/29
22	22		Ha Noi	+	+	Mvi/HaNoi.VNM/50.2008/30

23	19	Hai Ba Trung	Ha Noi	+	+	Mvi/HaNoi.VNM/50.2008/42
24	13	Nho Quan	Ninh Binh	-	+	RVi/NinhBinh.VNM/20.2008/1
25	6	Nho Quan	Ninh Binh	-	+	RVi/NinhBinh.VNM/20.2008/2

In 2009, 26 measles virus isolates were obtained from 97 throat swab specimens: 1 from Nho Quan disitrict, Ninh Binh province; 1 from Tho Xuan district, Thanh Hoa; 7 from Ha Noi, they located in Dong Da district (1), Thanh Xuan district (1), Ba Dinh district (1), Hoan Kiem (1), Hoang Mai (1), Cau Giay(1) and 1 unknown location; 5 cases were from districts of Bac Giang province: Bac Giang town (2), Tan Yen (2), Luc Nam (1). 4 cases from Hai Duong province with one was from Chi Linh, Tu Ky, Thanh Mien district and Hai Duong City; 5 cases were from Nghe An province (Vinh City: 3 and Dien Chau district: 2); 1 cases were from Thanh Liem district of Ha Nam; 1 cases located in Kien Xuong district of Thai Binh province and 1 cases from Hoa Binh province.

Figure 1. Distribution of measles confirmed cases



Year 2006	Year 2008	Year 2009

Table 3: Description of measles and rubella viruses in 2009

Patient No.	Patient Age	Address		Result		Strain name
		District	Province	Virus isolation	RT-PCR	
26	22	Tan Yen	Bac Giang	+	+	MVi/BacGiang.VNM/8.2009/57
27	22	Tan Yen	Bac Giang	+	+	MVi/BacGiang.VNM/8.2009/58
28	17	Bac Giang	Bac Giang	+	+	MVi/BacGiang.VNM/9.2009/72
29	27	Luc Nam	Bac Giang	+	+	MVi/BacGiang.VNM/6.2009/17
30	41m	Bac Giang	Bac Giang	+	+	MVi/BacGiang.VNM/8.2009/58
31	19	Thanh Liem	Ha Nam	+	+	MVi/HaNam.VNM/6.2009/9
32	24	Cau Giay	Ha Noi	+	+	MVi/HaNoi.VNM/6.2009/2
33	23	Unknown	Ha Noi	+	+	MVi/HaNoi.VNM/7.2009/46
34	23	Thanh Xuan	Ha Noi	+	+	MVi/HaNoi.VNM/7.2009/47
35	25	Ba Dinh	Ha Noi	+	+	Mvi/HaNoi.VNM/7.2009/50
36	20	Hoang Mai	Ha Noi	+	+	MVi/HaNoi.VNM/7.2009/52
37	16	Dong Da	Ha Noi	+	+	MVi/HaNoi.VNM/7.2009/24
38	13	Hoan Kiem	Ha Noi	+	+	MVi/HaNoi.VNM/7.2009/25
39	26	Chi Linh	Hai Duong	+	+	MVi/HaiDuong.VNM/8.2009/75
40	19		Hai Duong	+	+	MVi/HaiDuong.VNM/8.2009/77
41	20	Thanh Mien	Hai Duong	+	+	MVi/HaiDuong.VNM/7.2009/8
42	24	Tu Ky	Hai Duong	+	+	Mvi/HaiDuong.VNM/7.2009/13
43	unknown	unknown	Hoa Binh	+	+	MVi/HoaBinh.VNM/8.2009/69

44	4	Dien Chau	Nghe An	+	+	MVi/NgheAn.VNM/44.2009/102
45	7	Vinh city	Nghe An	+	+	MVi/NgheAn.VNM/45.2009/103
46	5	Vinh city	Nghe An	+	+	MVi/NgheAn.VNM/44.2009/104
47	7	Vinh city	Nghe An	+	+	MVi/NgheAn.VNM/45.2009/105
48	4	Dien Chau	Nghe An	+	+	MVi/NgheAn.VNM/45.2009/106
49	19	Nho Quan	Ninh Binh	+	+	MVi/NinhBinh.VNM/12.2009/100
50	20	Kien Xuong	Thai Binh	+	+	MVi/ThaiBinh.VNM/6.2009/4
51	22	Tho Xuan	Thanh Hoa	+	+	MVi/ThanhHoa.VNM/12.2009/99
52	17	Tan Yen	Bac Giang	-	+	RVi/BacGiang.VNM/7.2009/36
53	59	Bac Giang	Bac Giang	-	+	RVi/BacGiang.VNM/8.2009/38
54	23	Tan Yen	Bac Giang	-	+	RVi/BacGiang.VNM/7.2009/39
55	6	Tan Yen	Bac Giang	-	+	RVi/BacGiang.VNM/8.2009/55
56	5	Tan Yen	Bac Giang	-	+	RVi/BacGiang.VNM/8.2009/59
57	22	Tan Yen	Bac Giang	-	+	RVi/BacGiang.VNM/8.2009/61
58	40	Tan Yen	Bac Giang	-	+	RVi/BacGiang.VNM/8.2009/62
59	33	Tan Yen	Bac Giang	-	+	RVi/BacGiang.VNM/8.2009/64
60	10	Tan Yen	Bac Giang	-	+	RVi/BacGiang.VNM/7.2009/65
61	5	Lang Giang	Bac Giang	-	+	RVi/BacGiang.VNM/9.2009/70
62	20	Lang Giang	Bac Giang	-	+	RVi/BacGiang.VNM/10.2009/96
63	25	Tan Yen	Bac Giang	-	+	RVi/BacGiang.VNM/7.2009/21

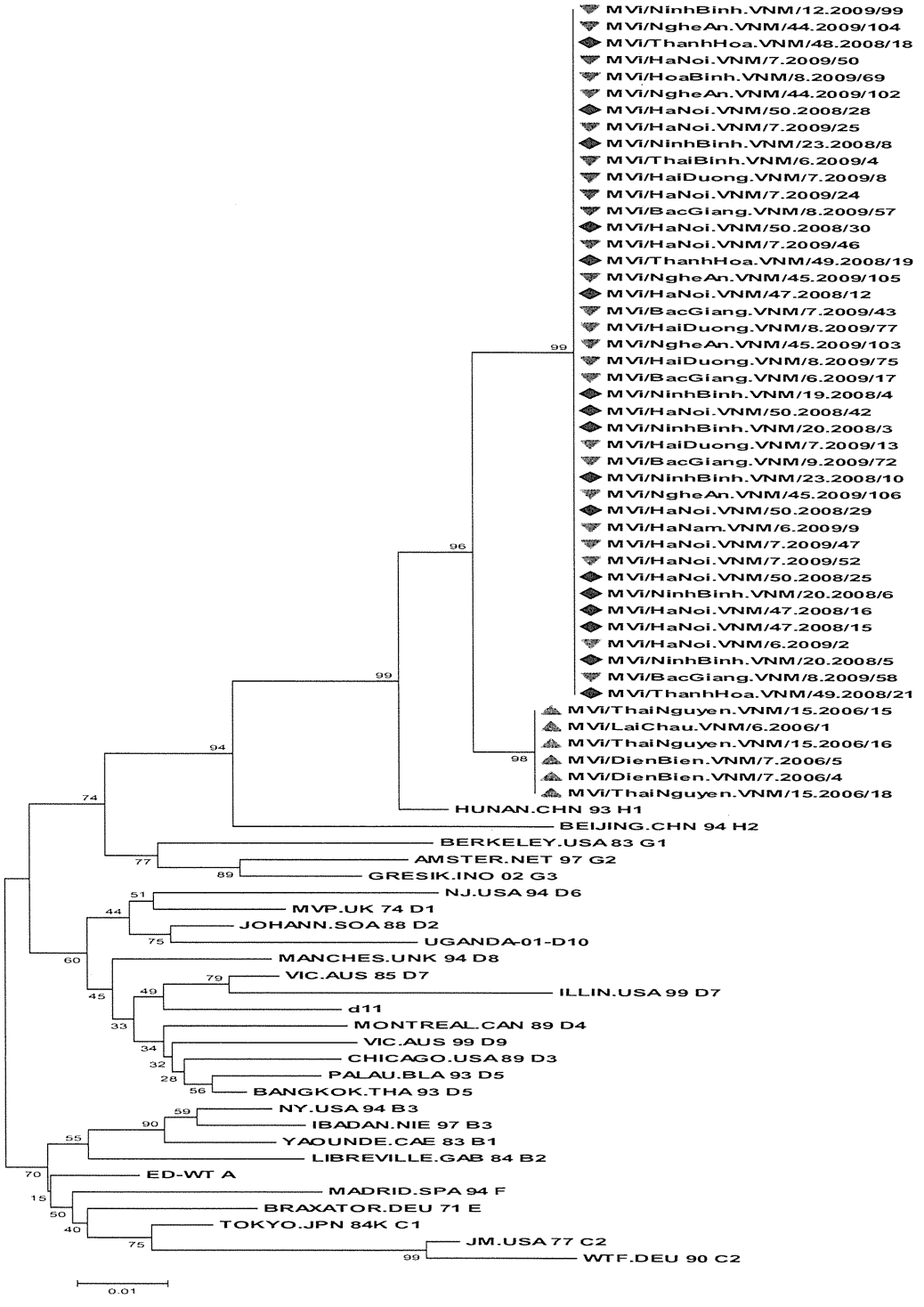
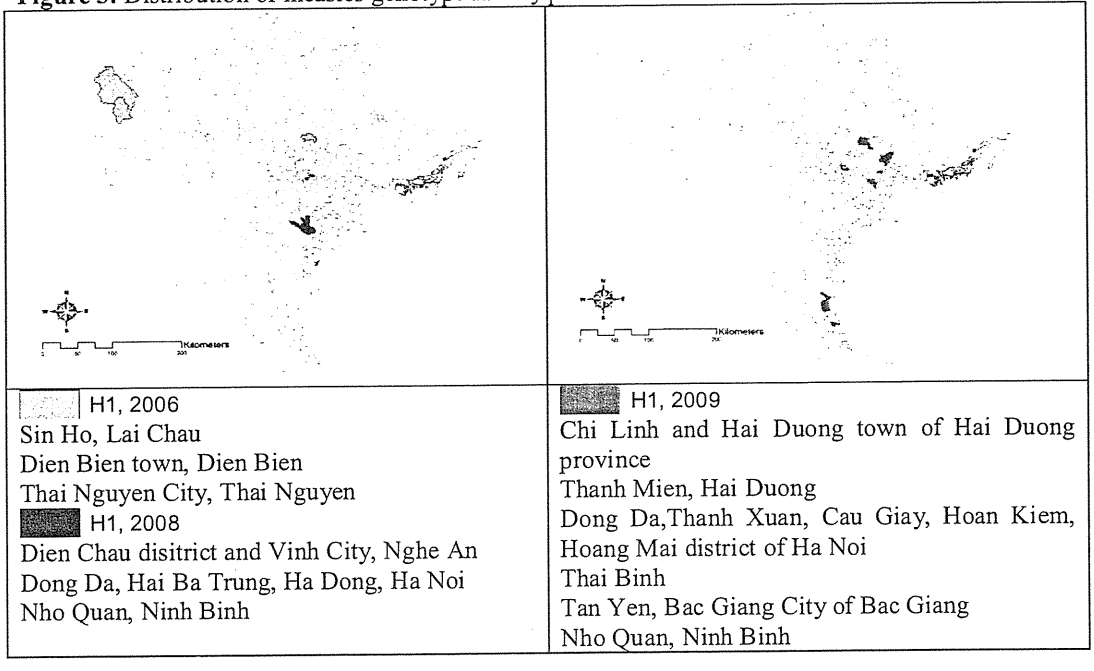


Figure 2. Phylogenetic analysis of the sequences of the nucleoprotein genes (450 nt) of the strains of measles virus from 2006-2009 in the North Vietnam. The unrooted tree shows sequences from Vietnam viruses compared with World Health Organization (WHO) reference strains for each genotype. MVi, measles virus sequence from isolates.

The phylogenetic tree is shown that the strains of MV in 2008-2009 in the North Vietnam grouped with the H1 reference sequences. The nucleotide (nt) difference between the Vietnamese strains in 2006 ranged from 0.2% to 0.6%. The nt difference between the Vietnamese strains in 2006 and H1 reference sequences in 2006 ranged from 2% to 2.4%. The nt difference between the Vietnamese strains in 2008 ranged from 0.2% to 0.4%. The nt difference between the Vietnamese strains in 2008 and H1 reference sequences in 2006 ranged from 2.4% to 2.7%. The nt difference between the Vietnamese strains in 2009 ranged from 0.2% to 0.6%. The nt difference between the Vietnamese strains in 2009 and H1 reference sequences ranged from 2.4% to 2.9%. The nt difference between the Vietnamese strains in 2008 -2009 ranged from 0.2% to 0.6%. The nt difference between the Vietnamese strains in 2008 – 2009 and 2006 ranged from 1.7% to 2.7%.

Figure 3. Distribution of measles genotype H 1 by place and time



H1 was the pathogen of 3 measles outbreaks in 2006. The outbreaks in Sin Ho district (Lai Chau province) and Dien Bien town (Dien Bien province) were closely related in place and time. The two districts were adjunction and the outbreaks dated both in February 2006. H1 caused several outbreaks in 2008. Three outbreaks in districts of Ha Noi (i.e. Dong Da, Hai Ba Trung, Ha Dong) were generally related, they were occurred from August to December 2008. In Nho Quan district of Ninh Binh, H1 caused an outbreak in May 2008. It caused one outbreak in Sam Son district of Thanh Hoa province in January - February 2008. H1 continually circulated causing outbreaks in 2009. H1 caused two outbreaks in Hai Duong province. The first one occurred in Chi Linh district and Hai Duong City in February 2009. The second one located in Thanh Mien district in September. It caused three outbreaks in districts of Ha Noi City dating February, June and October 2009. It was noticed that H1 came back to Dong Da district causing another outbreak in 2009 following the 2008 outbreak. Similarly, it caused the second outbreak in Nho Quan district of Ninh Binh province. An outbreak in Bac Giang City and Tan Yên district of Bac Giang province and outbreak in Thai Binh dated February 2009.

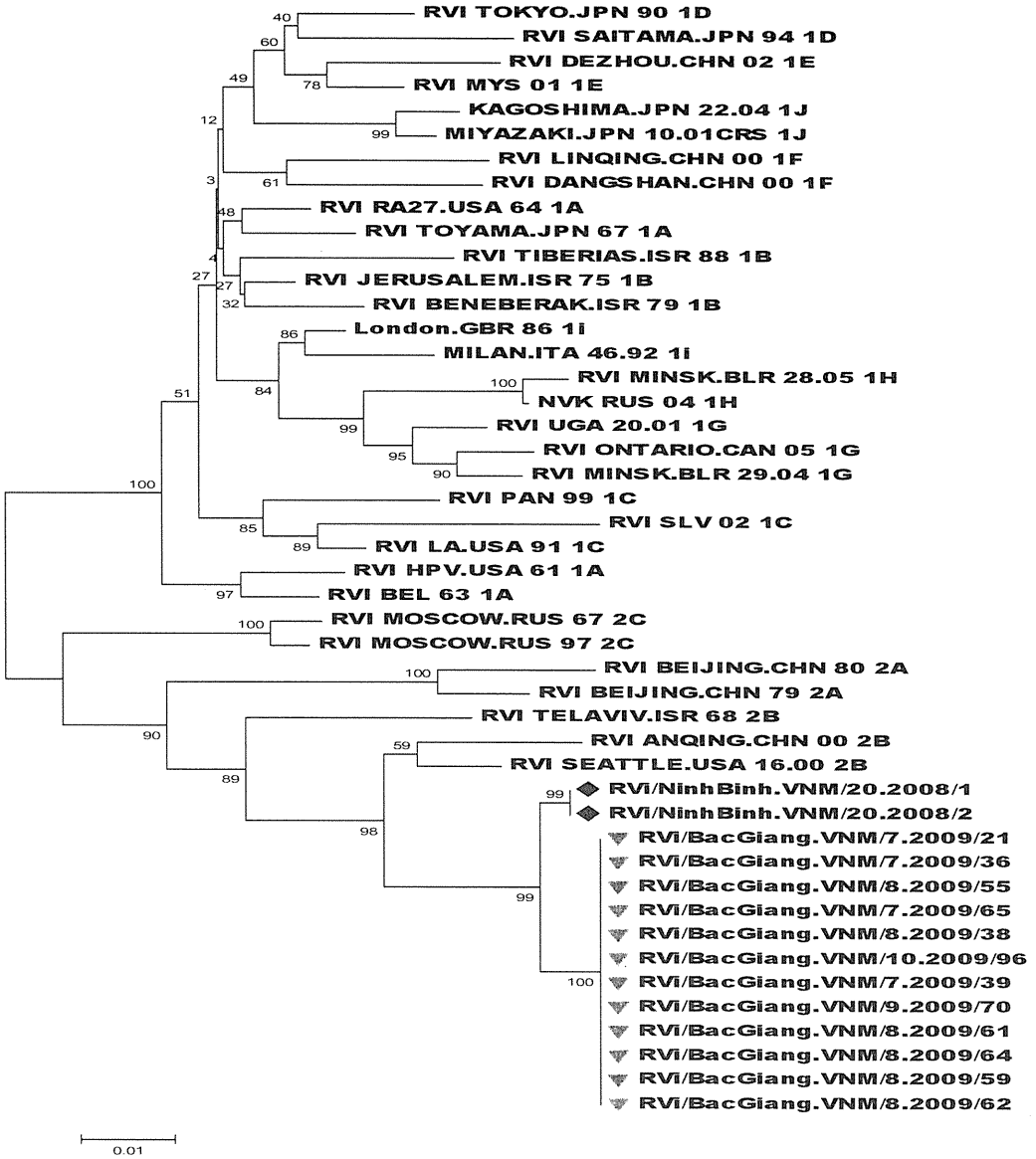


Figure 4. Phylogenetic analysis of the sequences of the E1 gene (739nt) of the strains of rubella virus from 2008-2009. The unrooted tree shows sequences from Vietnam viruses compared with World Health Organization (WHO) reference strains for each genotype. Rvi, rubella virus sequence from isolates.

The phylogenetic tree of RV is shown that the strains of RV in 2008-2009 in the North Vietnam grouped with the 2B reference sequences. The nt difference between the Vietnamese strains in 2008 and 2B reference ranged from 2.8% to 6%. The nt difference between the Vietnamese strains ranged from 0.8% to 1.4%.